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Renal entactin (nidogen): Isolation, characterization and tissue distribution

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Renal entactin (nidogen): Isolation, characterization and tissue distribution. Entactin/nidogen (E/N) was isolated from bovine renal tubular basement membrane. Apparent molecular weight, amino acid composition, and molecular configuration by electron microscopy rotary shadowing were similar to that of nidogen from EHS mouse tumor. The identity of bovine E/N was confirmed using a thrombin derived peptide, the sequence of which corresponded to a region within mouse and human E/N. Monoclonal and polyclonal anti-E/N antibodies were used to determine the distribution of E/N in human kidney by immunofluorescent and immunoelectron microscopy. E/N was present in all renal basement membranes and was distributed through the full width of the glomerular basement membrane (GBM) with accentuation along its epithelial aspects. E/N distribution was similar to that of novel collagen chain α3(IV) NC domain in the GBM. In the mesangium, E/N was distributed mainly in the peripheral mesangial region that is bounded by the GBM, while classical collagen chain α1(IV) NC as present diffusely throughout the mesangium. In the developing nephron, E/N was present in basement membranes of the ureteric bud, primitive vesicle and S-form. In all instances, E/N co-localized with laminin B2 chain. Prominent E/N detection within the mesangium was observed in diseases where mesangial expansion was present. This process was also seen in early diabetic nephropathy, but disappeared with disease progression. However, all thickened diabetic renal basement membranes showed an increase in E/N which was also present in Kimmelstiel-Wilson lesions. E/N was observed in the GBM "spikes" of membranous glomerulonephritis and in epithelial crescents associated with various disorders. The association between E/N, laminin and type IV collagen chains observed in the normal kidney were maintained in disorders with altered E/N distribution. We could not detect any changes in the distribution of E/N in other acquired and hereditary kidney diseases. These observations reflect the involvement of E/N in the structure and disease alteration of renal basement membranes and mesangial matrix.

Basement membranes are extracellular matrices which support vascular and epithelial cells, maintain tissue architecture, and serve as barriers to macromolecules and migrating cells. This molecular scaffold is composed of type IV collagen which forms large cross-linked networks. Associated non-collagenous proteins include laminin, proteoglycans, and entactin/nidogen (E/N) [1]. The latter glycoprotein was isolated from the mouse EHS tumor and shown to consist of two globular domains attached by a rod-like structure creating a dumbbell shaped molecular [2, 3]. Nidogen is closely associated with laminin [4], binds to type IV collagen [5], and modulates cell binding to laminin [6]. The cDNA sequence for mouse nidogen has established an identity with entactin, a glycoprotein isolated from extracellular matrix of a mouse embryo cell line [7, 8]. Neither nidogen nor entactin have been isolated and characterized from an intact basement membrane.

Immunohistochemical studies have shown E/N to be part of basement membranes of various organs. Its distribution in renal extracellular matrices has been examined in rodents using immunochemical techniques [2, 9–14].

We report the isolation and characterization of E/N from bovine renal tubular basement membrane. Its distribution in human kidney was studied by immunohistochemical techniques using monoclonal and affinity-purified polyclonal antibodies. We describe the relationship to laminin (B1 and B2 chains), and type IV collagen in normal human kidney, in various renal disorders and during renal ontogeny.

Methods

Nidogen purification

Bovine kidney tubular basement membranes were prepared as previously described [15]. Cortical tissue was dissected to exclude medullary elements. Aliquots were suspended in a solution containing ethylenediamine-tetracetic acid, epsilon amino caproic acid, N-ethylmaleimide, and phenyl-methylsulfonyl fluoride protease inhibitors [15]. Following tissue disruption using a polytron homogenizer, the suspension was successively passed over metal sieves. Material retained on a #170 sieve was sonicated and centrifuged. The resultant pellet was washed with 1.0 M NaCl and distilled water, and then lyophilized.

The crude basement membrane material was extracted overnight with 6.0 M guanidine HCl, 50 mM phosphate pH 7.4, containing protease inhibitors, as above [15]. The extracts were dialyzed extensively against 6.0 M urea 50 mM phosphate pH 7.4 and subjected to ion exchange chromatography on DEAE-cellulose in 6.0 M urea at 20°C. The column was eluted with a 0.0 to 0.8 M NaCl linear gradient. Column fractions containing E/N were identified by SDS-PAGE, pooled, concentrated,

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dialyzed against 6.0 M urea 0.02 M Na acetate pH 4.8, and passed over an S-Sepharose column at 20°C. Elution was performed using a linear 0.0 to 0.4 M NaCl gradient, and fractions containing E/N were pooled and dialyzed in guanidine for further purification by Sephacryl S-300 gel filtration. For final purification, E/N obtained from gel filtration pools was applied to a Mono Q column in 0.05 M Tris pH 7.4 and eluted in a 0.0 to 0.5 M NaCl gradient.

Nidogen characterization and analysis

E/N in column fractions was identified by SDS-PAGE using 5 to 15% gradient slab gels [16]. Molecular weight determination was made using globular protein standards (Sigma Chemical Co., St. Louis, Missouri, USA), including carbonic anhydrase, egg albumin, bovine albumin, phosphorylase B, beta galactosidase and myosin for calibration. EHS nidogen used as standard was provided by Dr. Aris Charonis (University of Minnesota, Rochester, Minnesota, USA). The presence of E/N in column fractions and protein pools was verified by Western blotting [17] using a semi-dry blot apparatus and rabbit antiserum to mouse tumor EHS-derived nidogen (provided by R. Timpl, Max Planck Institute for Biochemistry, Martinsried, Germany).

Amino acid analysis was performed on 25 to 50 μg of protein hydrolyzed for 24 hours at 110°C in constant boiling HCl, and analyzed on a Beckman 6300 amino acid analyzer using a ninhydrin detection system. Intact E/N was incubated with thrombin (0.1 U/mg protein, Sigma Chemical Co.) for 12 hours at 37°C. Digestion fragments were purified by chromatography on a TSK G 3000 SW Ultropac column in 4.0 M guanidine HCl 25 mM phosphate pH 7.4. Selected peptides were then isolated on a 0.4 × 15 cm Vydac C4 reverse-phase HPLC column. Protein sequence determination of these peptides was done on an Applied Biosystem Sequenator, using automated Edman degradation. Amino acids were detected as PTH derivatives. Amino acid analysis and sequence determination were performed in the Microchemical Facility, Institute of Human Genetics, University of Minnesota.

Rotary shadowing was performed on E/N samples in bicarbonate buffer as previously described [18]. Samples were shadowed under vacuum with a mixture of 95% platinum 5% carbon in a Balzers apparatus and then examined with a Philips 300 transmission electron microscope operating at 60 KV (performed by Drs. E. Tsilibary and A. Charonis, University of Minnesota).

Immunologic methods

Polyclonal antibodies to E/N were made by immunizing rabbits with purified bovine E/N. Affinity column purified antibodies were prepared using E/N coupled to affinity matrix discs (Nalge Company, Rochester, New York, USA). Bound antibodies were eluted with 0.1 M glycine pH 2.5. To further ensure antibody specificity, E/N was transferred from SDS-PAGE gels to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, Massachusetts, USA) by semi-dry electrophoresis transfer. A strip containing E/N was cut from the transfer membrane and reacted with antibodies previously purified by affinity column chromatography as described above. Antibody was then eluted from the strip using 0.1 M glycine pH 2.5 and neutralized [19].

A monoclonal antibody to human E/N, Mab A9, was developed by immunizing mice with the collagenase resistant residue of human GBM, using standard procedures [20]. Reactivity of the monoclonal antibody was tested by Western blotting with preparations of purified E/N and with 6.0 M guanidine HCl and SDS extracts of human and bovine TBM. Monoclonal antibodies MBM 102 and MBM 17 to human α1(IV) and α3(IV) globular domains of classical and novel type IV collagen chains, respectively [21, 22], and 4E10 to laminin B1 chain (Dr. Eva Engvall) and 2E8 to laminin B2 chain (Developmental Studies, Hybridoma Bank, Johns Hopkins University) were also used [23].

Indirect immunofluorescent analysis was performed as previously described, using fluorescein labeled secondary antibodies [23]. Cortical slices of human and bovine kidneys were snap frozen in isopentane precooled in liquid nitrogen. Two micron sections of frozen tissue were obtained and processed for immunohistochemical analysis. Antibodies were tested on frozen tissue sections that were processed in the conventional way and also following tissue denaturation by incubation in acid-urea [24]. Orientation and spatial distribution within basement membranes was analyzed by high resolution phase-contrast microscopy and epifluorescence. Dual antibody staining was performed with appropriate controls as previously described [22, 25] using in sequence mouse monoclonal anti-E/N, rhodamine-conjugated goat antimouse IgG, rat anti-α1(IV) NC, and fluorescein-conjugated goat anti-rat IgG. Similarly, when the tissue distribution of E/N and α3(IV) NC domain was compared, tissue sections were first reacted with mouse monoclonal anti-α3(IV) NC followed by rhodamine conjugated goat anti-mouse IgG, and then with affinity-purified rabbit anti-E/N followed by fluorescein-conjugated goat anti-rabbit IgG. All secondary antibodies were appropriately absorbed to avoid cross reactive IgG binding. Control sections were stained with absorbed secondary antibodies alone.

E/N distribution was determined in normal human and bovine kidneys, during ontogeny using sections of human fetal kidney of 16 to 22 weeks gestation, and in three to six patients with each of the following disease states: IgA nephropathy, anaphylactoid purpura nephritis, membranoproliferative glomerulonephritis type I and II, lupus nephritis, membranous glomerulopathy, poststreptococcal glomerulonephritis, diabetic nephropathy, familial (Alport) nephropathy, thin membrane disease, autosomal-recessive polycystic kidney disease, minimal change nephrotic syndrome, congenital nephrotic syndrome, and nephrotic syndrome with focal segmental sclerosis.

Immunoelectron microscopy

Small pieces of human and bovine kidneys were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde, 0.1% picric acid in 0.1 M Sorenson's phosphate buffer, or in periodate lysine paraformaldehyde fixative [26]. Samples were washed and dehydrated in a graded ethanol series at successively lower temperatures down to −35°C and infiltrated with Lowicryl K4M. Ultrathin silver-to-gold interference contrast sections were cut and mounted on formvar coated, 100-mesh nickel grids. The grids were then floated on drops of phosphate buffer saline containing 5% pre-immune serum of the animal in which the secondary antibody was made for 10 minutes at 20°C. Grids were then incubated overnight at 4°C with mouse monoclonal or rabbit polyclonal anti-E/N antibodies; control staining of grids with either normal mouse serum or normal rabbit serum was

routinely performed. This was followed by the application of affinity-purified anti-mouse or anti-rabbit IgG coupled to 10 nm colloidal gold particles for two hours at 20°C. The grids were then washed with phosphate buffered saline 1% bovine serum albumin and 0.05% Tween, and stained with uranyl acetate and lead citrate and examined with a JEOL-100CX electron microscope at 60 KV.

Results

Bovine E/N purification

Guanidine extracts of bovine tubular basement membrane were subjected to ion exchange chromatography on DEAE cellulose in 6.0 M urea. By SDS-PAGE analysis, E/N was eluted with few contaminating proteins in the ascending portion of the first peak corresponding to 0.2 M NaCl of the salt gradient (Fig. 1A). These fractions were pooled and applied to a column of S-Sepharose (Fig. 1B): samples from the major peak were concentrated, dialyzed against 6.0 M guanidine, and further resolved on a column of Sephacryl S-300 (not shown). Protein from the main peak was applied to a Mono Q column in the final purification step (Fig. 2). SDS-PAGE of the material eluted from the Mono Q column showed a single band with an apparent molecular weight of 168 kDa. This compares with a molecular weight of 175 kDa observed for EHS nidogen in these experiments. Figure 3 compares the mobilities of bovine tubular basement membrane E/N (lane 1) with EHS nidogen (lane 6). Bovine E/N reacted with the polyclonal antibody to EHS mouse tumor nidogen (not shown), and with the monoclonal and polyclonal anti-E/N antibodies by Western blotting (lanes 2 to 5).

Characterization of bovine nidogen

The amino acid composition of E/N derived from bovine tubular basement membrane shows a similarity to that described for EHS derived nidogen (Table 1). Minor differences were observed in the content of glycine which was reduced in concentration, while the amounts of valine and arginine were increased compared to EHS nidogen. We were not successful in obtaining an amino acid sequence of the intact form of purified bovine E/N using automated Edman degradation.

The thrombin digest of the intact form of E/N generated three major peaks on the TSK G 3000 SW HPLC column (Fig. 4A). Upon reduction, the major peak (100 kDa molecular weight) dissociated into two fragments which were separable by gel filtration on TSK G 3000 SW column (Fig. 4B). The N-terminal sequence of the smaller molecular weight fragment corresponds to that described for a 22 kDa fragment generated through thrombin digestion of EHS nidogen [27]. Except for a substitution of proline by serine in position 6, the observed sequence is identical to residues 1040 to 1051 in the amino acid sequence of human nidogen deduced from placental cDNA (Fig. 5) [28]. Rotary shadowing of the intact bovine E/N disclosed ultrastructural conformation similar to that previously reported for this molecule (Fig. 6).

Characterization of anti-E/N antibodies

Monoclonal anti-E/N antibody reacted with the intact form of E/N and also recognized a 100 kDa E/N fragment (Fig. 3, lanes 2, 3). Minor reactivity with lower molecular weight fragments

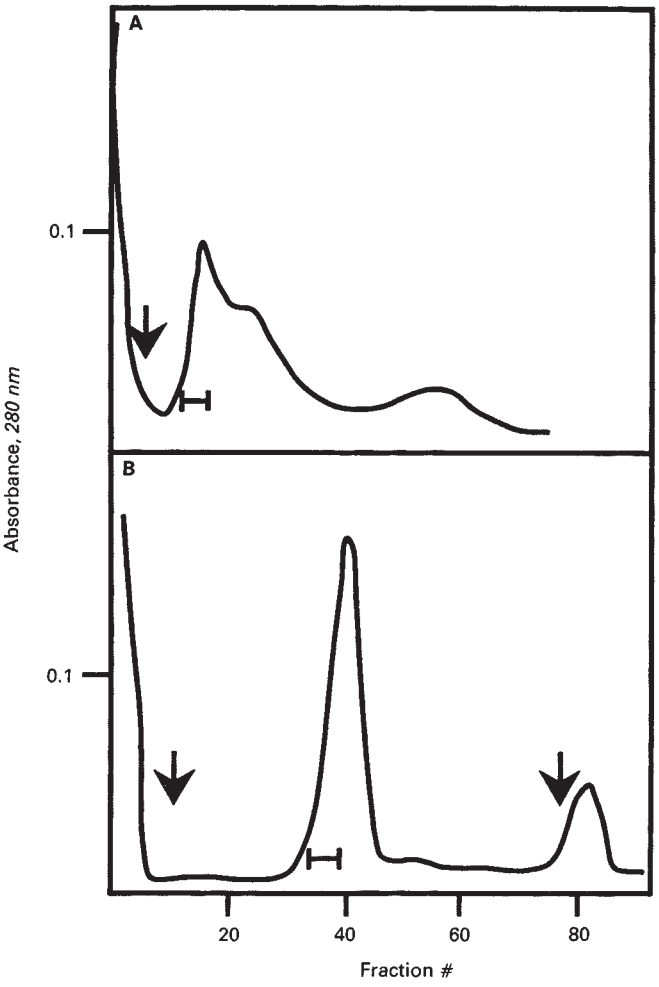

Fig. 1. *Fractionation of E/N by ion-exchange chromatography.* Bovine tubular basement membrane was extracted with guanidine and dialyzed into urea-phosphate buffer and subjected to chromatography on a column (2.5 × 15 cm) of DEAE-cellulose (**A**). Pooled fractions indicated by the bar in panel A were further purified on a column (2.5 × 15 cm) of S-Sepharose (**B**). Columns were developed with a 0.0 to 0.8 M (A) and 0.0 to 0.4 M (B) NaCl gradient followed by a final batch elution of S-Sepharose column with 1 M NaCl. Arrows indicate the start of the salt gradient and the batch elution. The bar in panel B indicates pooled fractions selected for further purification. Extraction and column buffers are given in **Methods**.

was also seen. The antibody reacted in ELISA assays and Western blots with E/N from bovine and human renal basement membranes, and EHS tumor. The polyclonal anti-E/N antibody showed reactivity similar to the monoclonal anti-E/N and also recognized trace quantities of 70 to 80 kDa E/N degradation products (Fig. 3, lanes 4, 5).

Tissue distribution of E/N

Affinity-purified rabbit anti-bovine E/N and monoclonal anti-human E/N antibodies reacted similarly with both human and bovine kidney, however, rabbit antibody to EHS tumor nidogen reacted only with bovine kidney. E/N was present uniformly in Bowman's capsule and the GBM. Diffuse staining of low intensity was observed throughout the mesangium with accentuation in the peripheral mesangial region which is bounded by

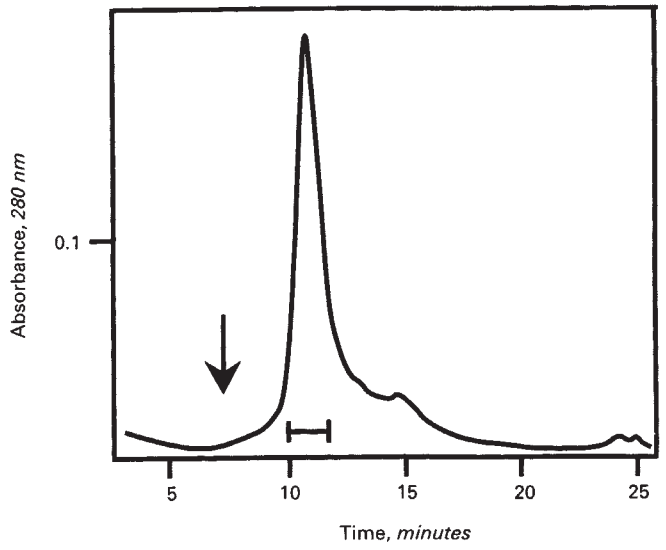

Fig. 2. *Preparation of nidogen by Mono Q chromatography.* Selected pools from a Sephacryl S-300 gel filtration column (not shown) were concentrated and applied to a Mono Q column in 0.05 M Tris, pH 7.4. The arrow indicates the beginning of a 0.0 to 0.5 M NaCl gradient, and pooled fractions shown by the bar contained the intact form of E/N used in subsequent studies.

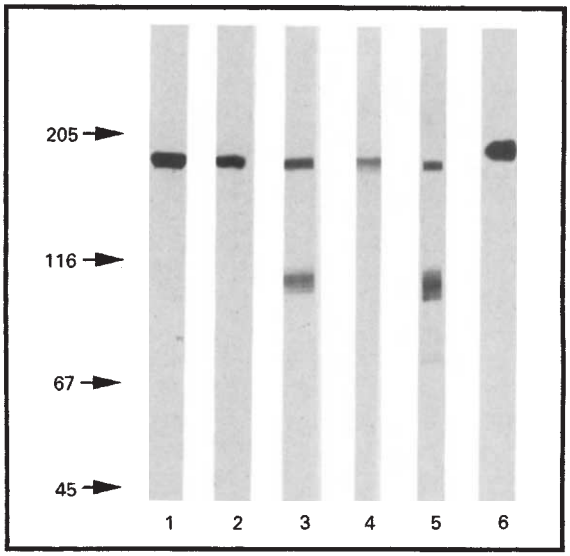

Fig. 3. *Electrophoresis and immunoblot analysis of E/N.* Purified E/N analyzed by SDS-PAGE and stained with silver is shown in lane 1. Western blots of pure bovine E/N using monoclonal (lane 2) and affinity-purified polyclonal anti-E/N antibodies (lane 4) demonstrate binding of these antibodies to the purified protein. Western blots of SDS-solubilized bovine tubular basement membrane with monoclonal (lane 3) and polyclonal (lane 5) anti-E/N antibodies reveal a major band corresponding in mobility to intact native E/N (lane 1) and a diffuse band of approximately 100 kDa that is less intense. Trace quantities of 70 to 80 kDa material in SDS-soluble tubular basement membrane representing E/N degradation products are identified with the polyclonal anti-E/N antibody (lane 5). Reactivity of the monoclonal antibody with EHS derived E/N (lane 6).

the reflection of the GBM (the waist area). GBM staining was single linear and, by phase fluorescence microscopy showed localization in the outer aspect of the GBM. Tubular basement membrane and basement membranes of vessel walls, including peritubular capillaries, were also stained (Fig. 7). Exposure of tissue sections with acid urea reduced the staining intensity of tubular basement membrane but did not alter that of the GBM. Similar distribution was observed for laminin B2 chain in the mesangium and other basement membranes while antibodies to laminin B1 chain only stained Bowman's capsule and weakly in the mesangium. Using dual-labeled antibody staining, E/N was observed to have a distribution similar to the putative α3(IV) NC domain of type IV collagen along the outer aspect of the GBM in a locus different from the subendothelial distribution of the α1(IV) NC of type IV classical collagen chains. In the mesangium α3(IV) NC collagen chains were absent, whereas E/N was present mainly in the region adjacent to the reflection of the GBM, while α1(IV) NC domain of classical type IV collagen was distributed diffusely throughout the mesangium (Fig. 8 A–F).

Immunoelectron microscopy confirmed these findings using both polyclonal and monoclonal anti-E/N antibodies. E/N was present in all basement membranes, in the mesangial matrix, and in the GBM preferentially along its outer epithelial aspect (Fig. 9).

We examined the distribution of E/N in the basement membrane structures of the nephron during ontogenesis (Fig. 10). Basement membranes of the ureteric bud, primitive vesicle, S-form and glomerulus in the early capillary loop stage were positive by indirect immunofluorescence. The distribution of laminin B2 chain was identical to that of E/N. Antibody staining for α1(IV) NC was found in all the above structures, while the novel chain α3(IV) NC appeared first in only the basement membranes of the early glomerular capillary stage of nephron development, as previously described [22].

Table 1. Amino acid composition of bovine tubular basement membrane entactin/nidogen

Amino acid	Species (residues /1000)	
	Bovine	EHS nidogen
Aspartic acid	96	99
Threonine	65	66
Serine	76	85
Glutamic acid	113	113
Proline	73	67
Glycine	94	105
Alanine	62	60
Half-cystine	37	32
Valine	73	57
Methionine	8	5
Isoleucine	40	37
Leucine	66	66
Tyrosine	31	36
Phenylalanine	42	36
Histidine	30	33
Lysine	30	31
Arginine	64	55

Results are an average of 4 determinations. The composition of EHS tumor nidogen is from Paulsson et al [3].

Distribution of E/N in renal disorders

Prominent mesangial staining for E/N was seen in all diseases in which mesangial expansion is a feature (systemic lupus

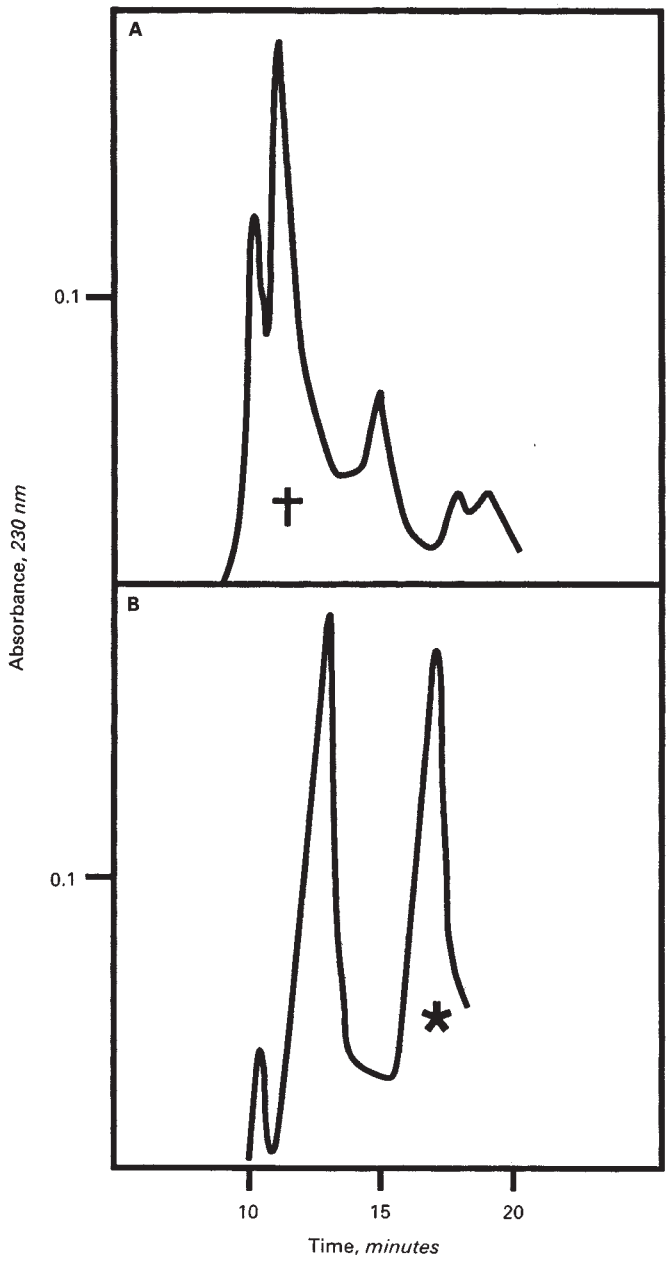

Fig. 4. *Purification of a thrombin-derived peptide of E/N.* E/N was digested with thrombin and applied to a TSK 3000 gel filtration column in 4.0 M guanidine (**A**). The major peak, indicated by the cross was reduced with mercaptoethanol and the products were resolved by gel filtration under the same conditions (**B**). The asterisk indicates the peptide that was subjected to chemical sequencing.

	1040											1050
Bovine	G	I	V	T	D	S	V	R	G	N	L	Y
Human	–	–	–	–	–	–	–	–	–	–	–	–
EHS tumor	–	–	–	–	–	P	–	–	–	–	–	–

Fig. 5. *Amino-terminal sequence of a 22 kDa thrombin digestion product of bovine tubular basement membrane E/N.* Protein sequence of E/N thrombin fragment (top line) is compared with homologous regions of humans [28] and mouse EHS tumor nidogen [27]. Complete identity was found between the sequence of the peptide and the cDNA derived sequence of human nidogen from amino acids 1040 to 1051 (numbering is according to the human sequence). One substitution (ser/pro) in this region is seen compared to the EHS tumor sequence.

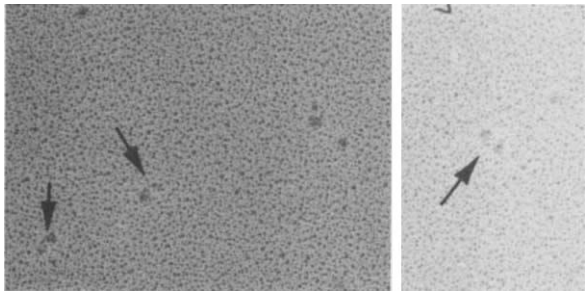

Fig. 6. *Electron micrographs of purified bovine E/N following rotary shadowing.* Selected fields are shown displaying the dumbell shape (arrows) characteristic of E/N (× 24,440).

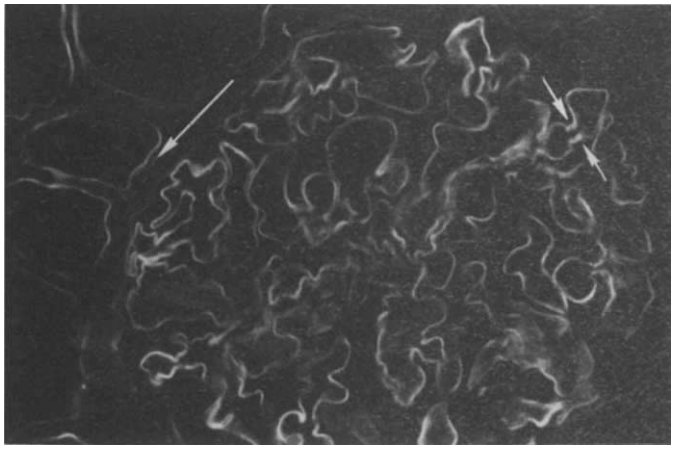

Fig. 7. *Immunofluorescent microscopy using monoclonal anti-E/N antibody on normal human kidney.* Reactivity is found in the glomerular basement membrane, Bowman's capsule, tubular basement membrane, and peritubular capillaries (arrow). In the mesangium E/N is detected primarily within the waist (double arrows) region (× 410).

erythematosus, IgA nephropathy, anaphylactoid nephritis and membranoproliferative glomerulonephritis type I and II) (Fig. 11). A double contour E/N staining of the split GBM was seen when mesangial interposition was present in type I membranoproliferative glomerulonephritis. Dense deposits present in type II membranoproliferative glomerulonephritis did not react with antibodies to E/N. Lacey staining with anti-E/N antibodies was seen in epithelial crescents and in the sclerotic lesions of focal segmental glomerular sclerosis. No staining for E/N was observed in the subepithelial immune deposits of poststreptococcal and membranous glomerulonephritis. In the latter E/N was present in the basement membrane "spikes" adjacent to the immune deposits along the epithelial aspect of the glomerular basement membrane (Kim Y, manuscript submitted).

E/N distribution appeared normal in the renal basement membranes of patients with Alport familial nephritis, thin basement membrane disease, congenital and idiopathic nephrotic syndrome, and in autosomal recessive polycystic kidney disease.

In diabetic nephropathy, E/N was increased in all thickened

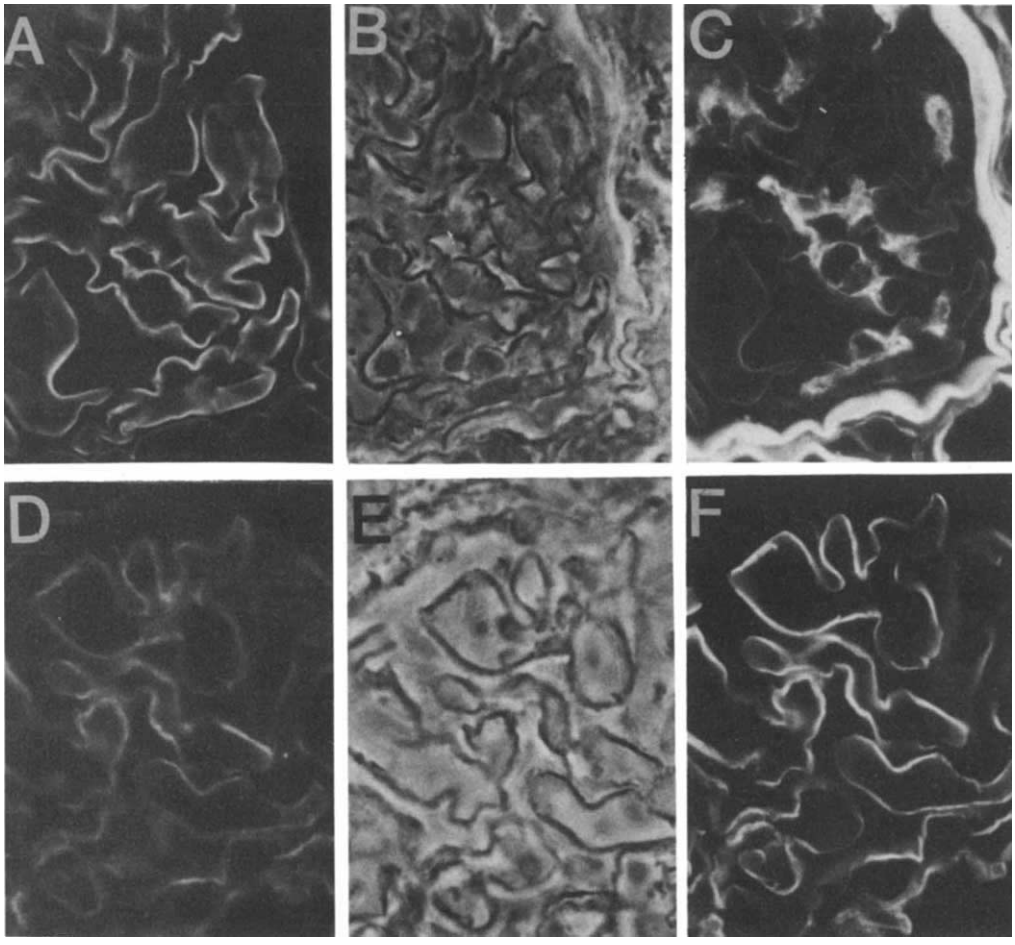

Fig. 8. *Immunofluorescent microscopy of normal human kidney using dual label antibody staining.* **A–C.** Monoclonal anti-E/N antibody (A), phase microscopy (B), and monoclonal anti-α1(IV) NC antibody (C). **D–F.** Polyclonal anti-E/N antibody (D), with phase microscopy (E), and anti-α3(IV) NC antibody (F) (× 623).

basement membranes (Fig. 12). In the early stages of diabetic mesangial expansion there was an increase in E/N staining of matrix material including Kimmelsteil-Wilson nodules. With progressive diabetic nephropathic change, E/N staining diminished in the mesangium; a similar pattern of α1(IV) NC and laminin B2 chain in the mesangium was found. Glomerular basement membrane staining for E/N was increased in all stages of diabetic nephropathy, and the same distribution and pattern of staining was seen with antibodies to laminin B2 chain. Increased glomerular basement membrane staining in all disease stages of diabetic nephropathy was also observed for the α3(IV) NC domain of novel chain type IV collagen [29].

Discussion

We report the isolation and characterization of E/N from bovine tubular basement membrane using methods similar to those described for EHS tumor derived nidogen [3]. We found that use of the S-Sepharose cation exchange column was more effective than CM cellulose, and a final HPLC anion exchange step (Mono Q column) was necessary to achieve satisfactory purification of the intact form of E/N.

E/N derived from bovine renal tubular basement was found to be similar to EHS derived nidogen in molecular weight, pattern of endogenous proteolysis, amino acid composition, and structure by rotary shadowing. The identity of the bovine E/N thus purified was supported by the partial amino acid sequence identity of a 22 kDa thrombin proteolytic product compared with the same region of human nidogen [27]. Our results are thus in agreement with a previous report of cDNA sequence similarity for human placental E/N and mouse tumor-derived nidogen [30].

We observed that E/N was present in all human renal basement membranes associated with vascular, tubular, and glomerular elements. In the glomerulus, E/N was observed in the GBM, mesangium and Bowman's capsule. E/N was present through the full width of the GBM but appeared to be increased along its epithelial aspect. Mesangial staining was light with accentuation in the mesangial waist area (Table 2).

The distribution of E/N corresponded closely to that seen for laminin B2 chain. A different staining pattern was observed for laminin B1 chain which was only detectable in Bowman's capsule and minimally within the mesangium, and was not found in the glomerular basement membrane [23]. In vitro binding studies have shown EHS derived E/N to bind either to the B1 chain of laminin [4], or to both B1 and B2 chains [31]. The above discrepancy could be attributed to the presence in

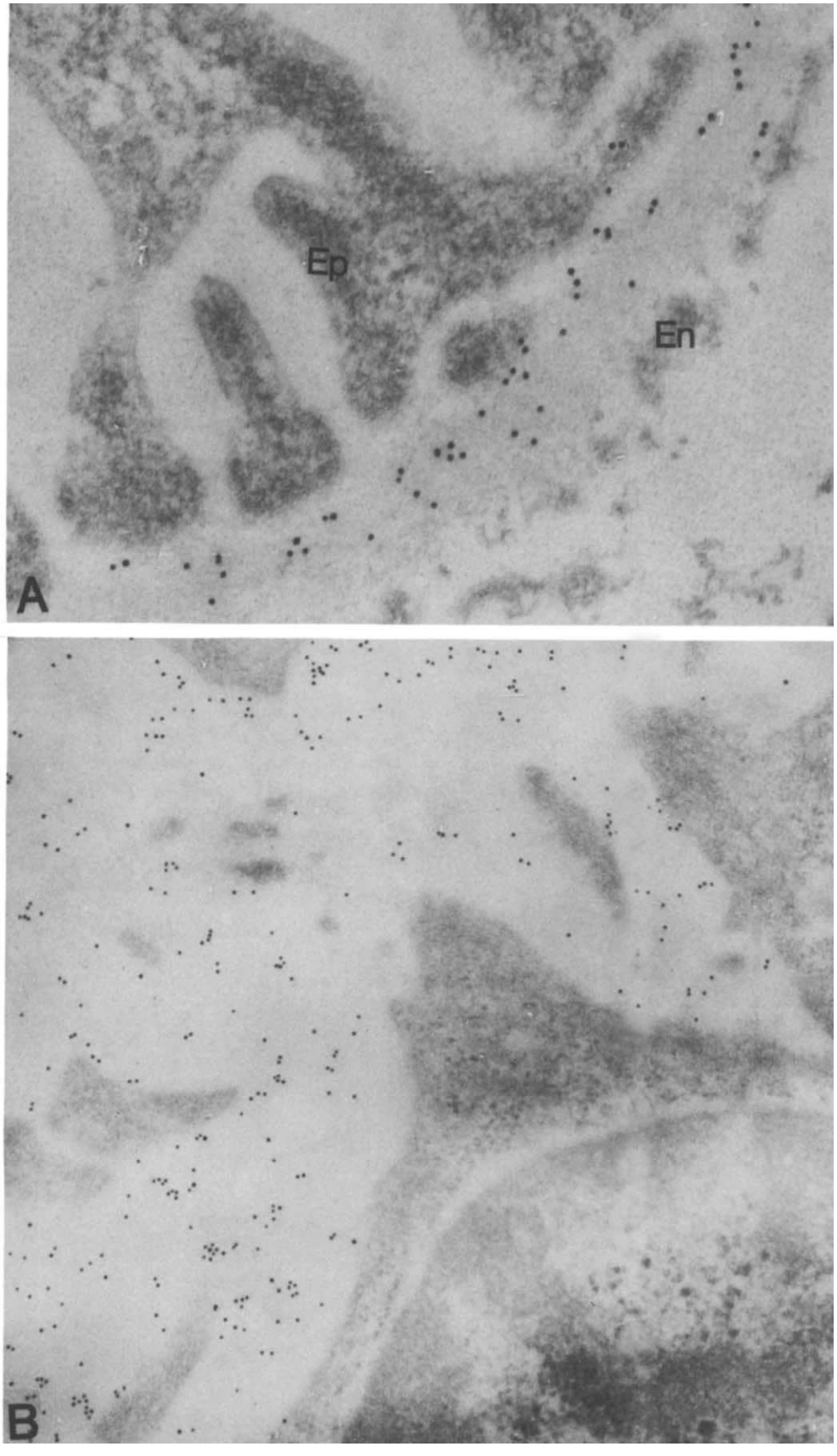

Fig. 9. *Immunoelectron microscopy of the glomerulus using the immunogold technique.* Immunoelectron microscopy of the glomerulus using the immunogold technique. Monoclonal anti-E/N antibody reactivity is localized to the outer aspect of glomerular basement membrane (**A**), and diffusely throughout the mesangium (**B**). Control staining with normal mouse serum was negative (× 46,500) (En = endothelium. Ep = visceral epithelium).

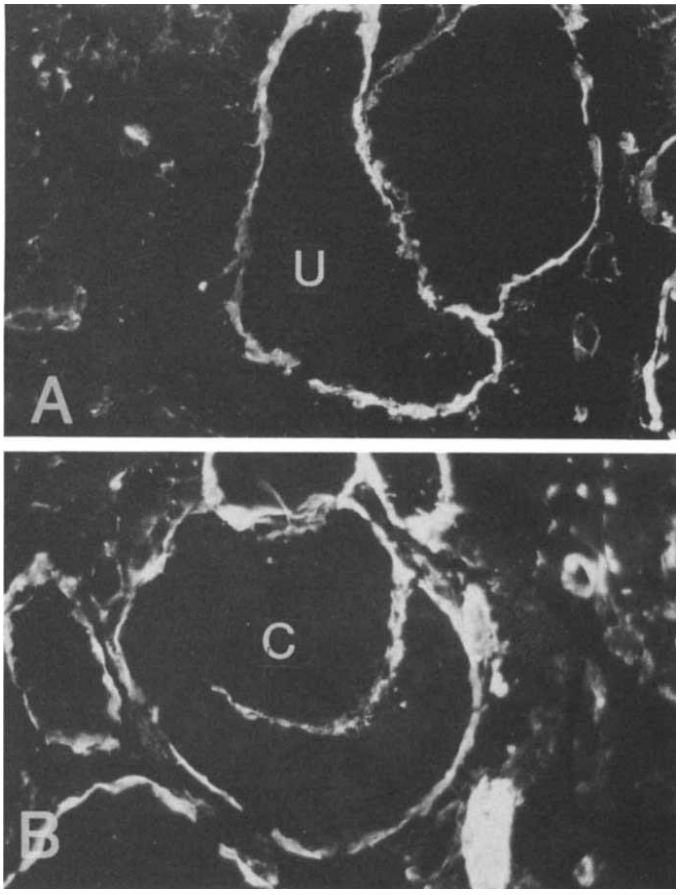

Fig. 10. *Immunofluorescent microscopy of normal human fetal kidney.* Monoclonal anti-E/N antibody reactivity is seen in the basement membranes of the ureteric bud (U) **(A)** and of the glomerular comma-form (C) **(B)** which were identified by phase microscopy (not shown) (× 410).

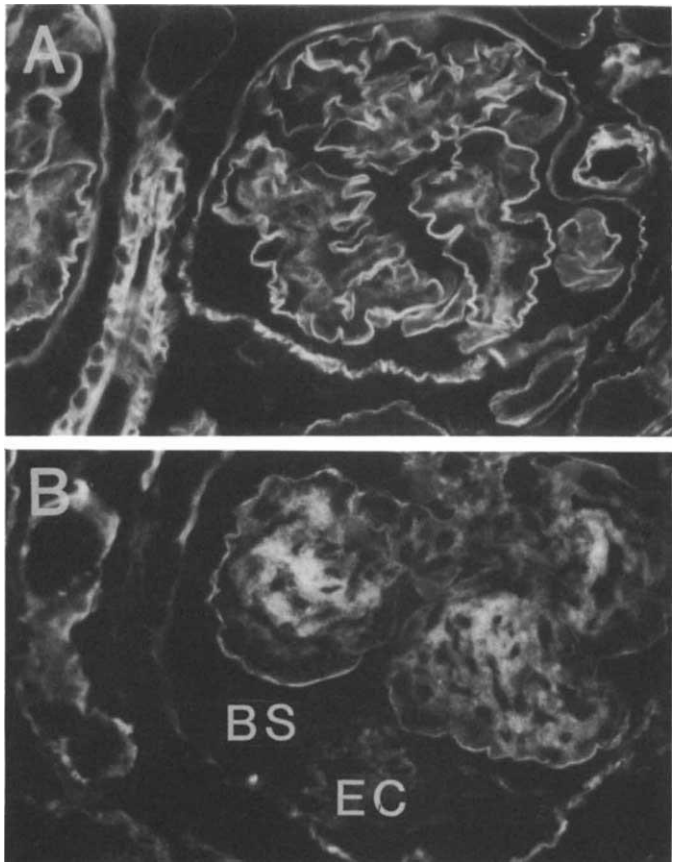

Fig. 11. *Immunofluorescent microscopy in type I membranoproliferative glomerulonephritis using monoclonal anti-E/N antibody.* Increased staining reactivity associated with mesangial proliferation **(A)**. Further mesangial expansion with glomerular lobulation, and lacey staining of an epithelial crescent (EC) identified by phase microscopy (not shown) within Bowman's space (BS) **(B)** (× 410).

human kidney of laminin isoforms with binding properties different than those of mouse laminin.

In the GBM, E/N distribution was similar to that of recently discussed novel chains of type IV collagen [tentatively identified as α3(IV) and α4(IV)] but differed from the distribution of the α1 and α2 type IV collagen which has a subendothelial localization. Although detected in different mesangial loci, E/N and the classical type IV chains are found in the mesangium, whereas the novel type IV chains are absent from this region (Table 2).

During renal ontogeny E/N distribution is similar to laminin B2 chain, but different from laminin B1 chain (not shown). Like E/N, classical type IV collagen chains are present early in ontogeny while the novel chains appear in the primitive glomeruli after vascular migration into the S-form [22]. E/N thus seems to be an active participant in the complex process of ontogenic development of the fused glomerular basement membrane. In the immunohistochemical studies of fetal, normal, and diseased human kidneys, the same staining patterns for E/N were observed using the monoclonal and rabbit anti-E/N antibodies.

E/N staining was increased in all diseases with mesangial expansion. When mesangial interposition is present in type I membranoproliferative glomerulonephritis, antibodies to E/N stain the glomerular basement membrane in a double linear pattern reflecting the presence of E/N in the lamina densa and in the interposed mesangial matrix. In membranous nephropathy E/N was present in the "spikes" surrounding the subepithelial immune complexes, likely reflecting increased synthesis by visceral epithelial cells, and similar to studies using antibodies to laminin and the novel collagen chains [Kim Y, et al, manuscript in preparation].

In diabetic kidney, E/N and laminin B2 chain were increased in all thickened basement membranes and remained altered in this fashion with progression of the disease. E/N and laminin B2 chain were present throughout the expanded mesangial matrix in early diabetic glomerulopathy but diminished in advanced nephropathy, whereas these components persisted in the GBM in advanced diabetic nephropathy similar to that observed for the novel chains of type IV collagen [29].

Previous reports have shown circulating anti-E/N antibodies in sera of patients with acute poststreptococcal glomerulonephritis [32], Goodpasture syndrome [33], and in experimental membranous glomerulopathy [34], suggesting a role for E/N in

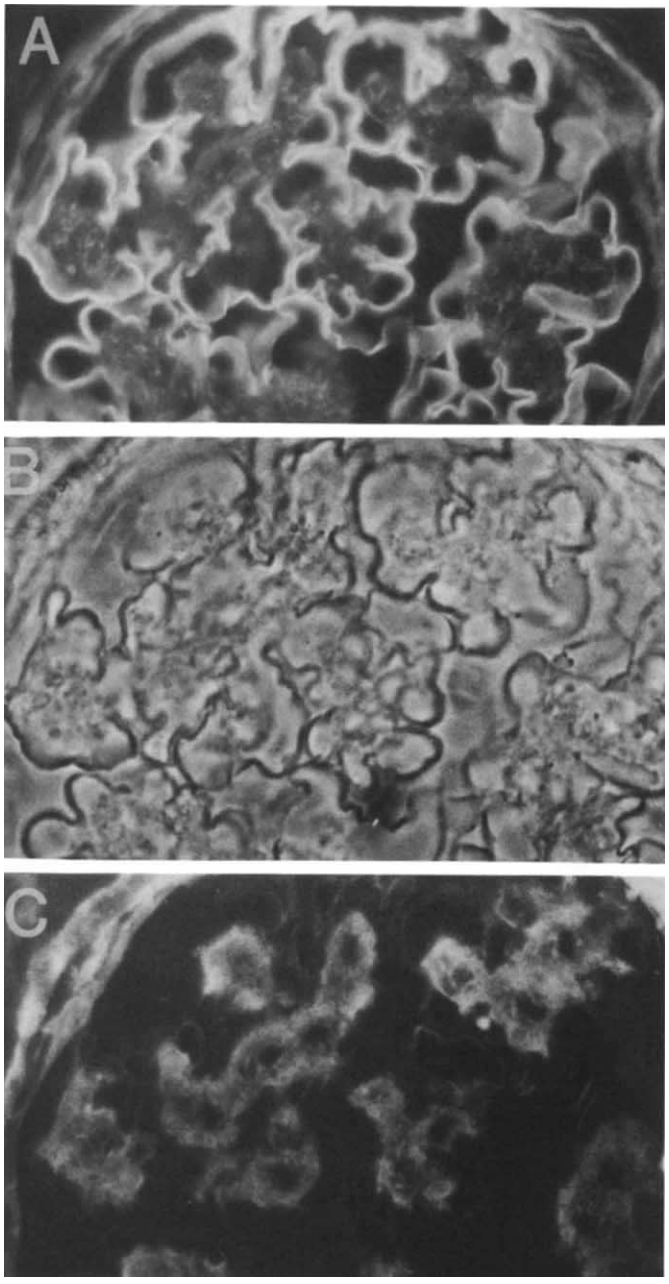

Fig. 12. *Immunofluorescent microscopy in diabetic nephropathy using dual-label antibody staining of the same glomerulus.* Polyclonal anti-E/N antibody (**A**), phase microscopy (**B**), and anti-α1(IV) NC antibody (**C**). Diffuse mesangial staining for E/N and classic type IV collagen chains is evident (× 576).

Table 2. Distribution of entactin/nidogen compared to other extracellular matrix components in normal human kidney by immunofluorescence

Location	Matrix components[a]			
	E/N	LM	α1(IV)	α3(IV)
Glomerulus				
Mesangium	+	+	+	−
Capillary subendothelium	−	−	+	−
Glomerular basement membrane	+	+	−	+
Tubule				
Tubular basement membrane	+	+	+	+
Vessels				
Arterioles	+	+	+	−
Capillary bed	+	+	+	−

[a] Matrix components are present (+) or absent (−). Abbreviations are: E/N, entactin/nidogen; LM, laminin B2 chain; α1(IV), NC domain type 1(IV) collagen chain; α3(IV), NC domain type 3(IV) collagen chain.

the pathogenesis of these immune complex disorders. Reactivity of the dense deposits seen in type II membranoproliferative glomerulonephritis [35] with wheat germ agglutinin could also suggest the presence of E/N since the latter reacts in vitro with wheat germ agglutinin [36]. Our findings do not support the presence of E/N either in the immune complexes of patients with acute poststreptococcal and membranous glomerulopathy, or in dense deposit material.

Since the observations of E/N distribution in various renal diseases are derived from limited numbers of patients with each disorder in this study, the following conclusions are tentatively proposed, pending more extensive study. The presence of E/N in all renal basement membranes and mesangial matrix in the mature kidney and all stages of renal ontogeny is an indication of its importance to renal structure. Furthermore, E/N distribution in basement membranes is not altered in disorders in which morphologic alterations of basement membrane structure has been observed (cystic kidney disease, Alport's familial nephritis, thin membrane disease) or when altered filtration permeability is present (idiopathic and congenital nephrotic syndrome) [37–39]. E/N co-localizes with laminin B2 chain while its relationship to type IV collagen varies from the mesangium (classical type IV) to glomerular basement membrane (novel type IV) collagen chains. These relationships are maintained in disease states where changes of mesangial proliferation are associated with increased E/N deposition along with laminin B2 and classic type IV collagen chains. However, GBM thickening is associated with increased deposition of E/N, laminin B2 and novel type IV collagen chains.

We propose that these changes reflect heightened synthetic capacity of glomerular mesangial cells in glomerular proliferative disorders, and glomerular epithelial cells in diabetic and membranous nephropathy, resulting in increased deposition of E/N in the mesangium and the glomerular basement membrane, respectively. Additional studies are needed to define synthesis of E/N by glomerular cell types and the factors which are responsible for their control.

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